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Millisecond studies of secretion in single rat pituitary cells stimulated by flash photolysis of caged Ca^{2+}

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To study the final steps in the secretory pathway of rat pituitary melanotrophs, we have monitored changes in cell surface area due to exocytosis after flash photolysis of caged Ca^{2+}. A step increase in cytosolic $[Ca^{2+}]$ to 45–125 μM triggers three phases of exocytic secretion. A small cohort of a few hundred vesicles is exocytosed in 40 ms in a secretory burst with a peak rate of 17 000 vesicles/s. Next, 2700 more vesicles are released in a slower phase that is complete within 400–1000 ms. Finally, vesicles continue to be released slowly (500 vesicles/s) for >8s. The approach described provides a way to identify and monitor the final steps in the secretory pathway at millisecond resolution. That a small portion of secretory vesicles can be released much faster than all others suggests that these vesicles are functionally equivalent to those at the presynaptic active zone of a neuron. Their release would be fast enough to be temporally correlated with single action potentials.

Key words: capacitance/DM-nitrophen/fura-2/furaptra/secretory pathway

Introduction

At a neuronal synapse, a small subset of the synaptic vesicles are docked directly beneath the plasma membrane at a so-called active zone and can be released in milliseconds after an increase in cytosolic Ca^{2+} (Kelly, 1991; Linstedt and Kelly, 1991). Endocrine cells have no morphologically recognizable active zones. Nonetheless, recent studies have suggested that endocrine cells also can release a small portion of their secretory vesicles very rapidly, while the remainder is released more slowly (Holz *et al.*, 1989; Thomas *et al.*, 1990; Augustine and Neher, 1992; Lindau *et al.*, 1992). In these studies, cytosolic $[Ca^{2+}]$, Ca_i, was raised by Ca^{2+} influx through the plasma membrane. This procedure causes large spatial Ca_i gradients in the vicinity of open Ca^{2+} channels (Roberts *et al.*, 1990). Therefore it is unclear whether rapid and slow phases of exocytosis arise from vesicles that have advanced to different stages in the secretory pathway, or because vesicles in different regions of a cell experience different Ca_i. To avoid this ambiguity, we have used a photolabile Ca^{2+} chelator, DM-nitrophen, (Kaplan and Ellis-Davies, 1988) to cause rapid and uniform increases in Ca_i, and have monitored exocytosis on a millisecond time scale by measuring the membrane capacitance (Neher and Marty, 1982; Fernandez *et al.*, 1983; Breckenridge and Almers, 1987). We observed three kinetically distinct phases of exocytosis in response to a step increase in Ca_i. Since these run their course even though Ca_i remains constant, they presumably reflect a sequence of late steps in the secretory pathway.

Results

To see how rapidly a melanotroph secretes in response to Ca^{2+}, we increased Ca_i by flash photolysis of Ca^{2+}–DM-nitrophen and monitored secretion by measuring the plasma membrane capacitance, an assay of cell surface area. Figure 1A shows a typical experiment. A fire-polished glass micropipette had been sealed against the plasma membrane and the membrane patch beneath its tip had been ruptured by a pulse of suction (Hamill *et al.*, 1981). Through the open tip, the solution in the pipette ('intracellular solution', see Materials and methods) had exchanged with the cytosol by dialysis. The solution also contained Ca^{2+}–DM-nitrophen, a photolabile Ca^{2+} chelator, and furaptra, a fluorescent Ca^{2+} indicator sensitive to Ca_i in the range 5–500 μM (Raju *et al.*, 1989; Konishi *et al.*, 1991; Lattanzio and Bartschat, 1991).

Figure 1A (top) tracks Ca_i, beginning after 118 s of dialysis. At this time, furaptra had filled the cytosol to equilibrium (as judged by the fluorescence of the cell), and the same may be assumed for Ca^{2+}–DM-nitrophen, a substance of closely similar molecular weight. Measurements with fura-2 (Almers and Neher, 1985; Grynkiewicz *et al.*, 1985) showed that Ca_i was low (163 ± 15 nM, mean ± SE, n = 4). We expect that cytosolic $[Mg^{2+}]$ was even lower since the total concentration of Mg in the pipette (< 10 μM) was at least 1000 times less than that of Ca, while DM-nitrophen chelates Mg^{2+} with 500-fold lower affinity than it does Ca^{2+} (Kaplan and Ellis-Davies, 1988).

After 120 s of dialysis (mean 149 s, range 97–235 s, n = 15), two flashes were given in succession. The first raised Ca_i 400-fold to 73 ± 7 μM (n = 11). This almost stepwise increase in Ca_i caused a secretory response (Figure 1A middle, C trace) that occurred in two phases. In the first phase, C increased by ~2000 fF in 1 s (1869 ± 94 fF, n = 16), while in the second it continued to increase almost linearly over the following 3 s (rate = 304 ± 23 fF/s, n = 16). Both effects are due to the increase in Ca_i, because in five otherwise identical experiments where the DM-nitrophen contained no Ca^{2+}, flash photolysis did not raise Ca_i and C did not change (not shown).

Although a second flash caused Ca_i to rise beyond 100 μM, the rise in C continued unchanged. Evidently the Ca^{2+}-dependence of this slower component is saturated at 60 μM. This component nonetheless requires Ca_i >1 μM,

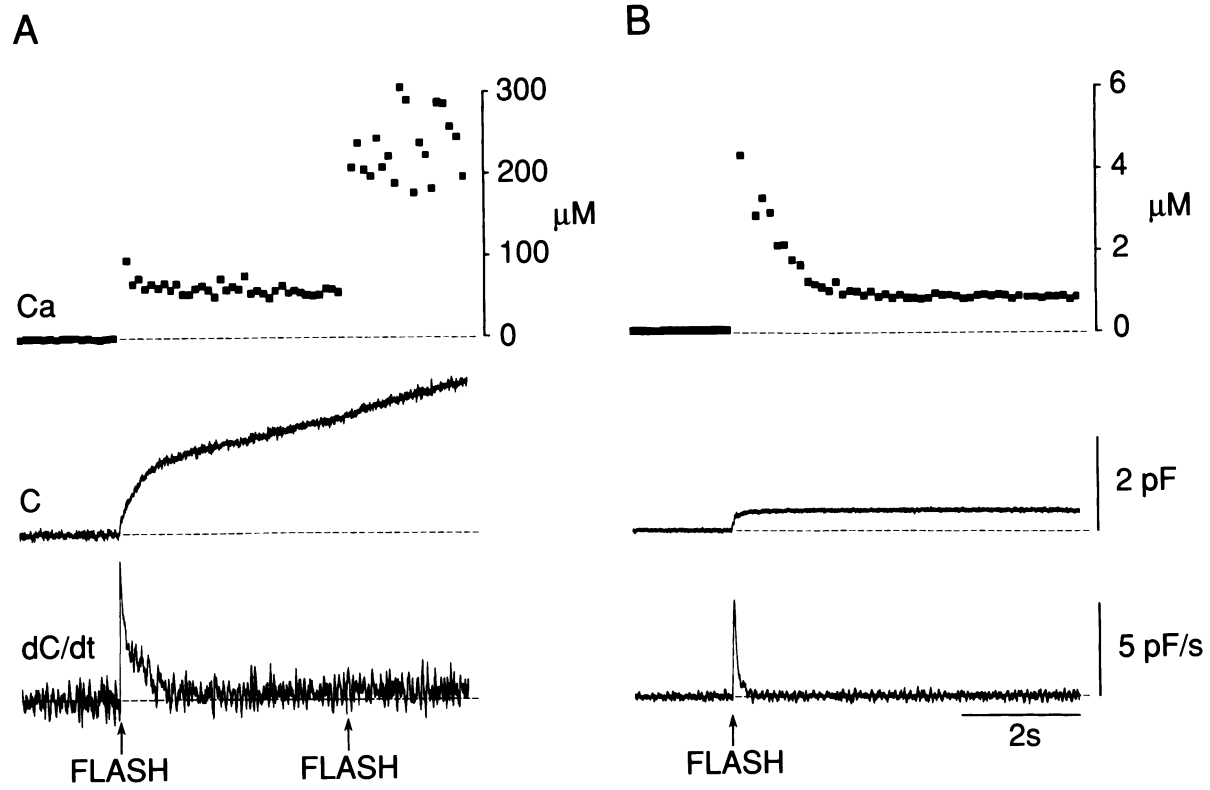

Fig. 1. Secretory responses to stepwise increases in Ca_i. From top to bottom, Ca_i (Ca), membrane capacitance (C) and its time derivative (dC/dt). dC/dt was determined by fitting straight lines to overlapping 50 ms segments of the C trace; the slopes of these lines were taken as dC/dt at the midpoint of each segment. (**A**) From a cell loaded through the patch pipette with intracellular solution containing 10 mM DM-nitrophen, 8.5 mM $CaCl_2$ and 0.1 mM furaptra. (**B**) From a cell loaded as in (A) except that a mixture of 5 mM $CaCl_2$ and 4 mM $MgCl_2$ replaced the 8.5 mM $CaCl_2$ and that the high affinity Ca^{2+} indicator fura-2 replaced furaptra.

because in cells where we replaced half of the Ca^{2+} in the DM-nitrophen cage with Mg^{2+} (Figure 1B) a more transient change in Ca_i was observed, and only a rapid phase of exocytosis was seen (amplitude 362 ± 35 fF, n = 8). In experiments with Mg^{2+}, Ca_i was well beyond the range sensed by fura-2 immediately after the flash and hence unknown.

The time derivative of C (dC/dt in Figure 1A bottom, proportional to the rate of secretion) suggests that this first phase can itself be subdivided into two components. The two phases are more clearly seen in Figure 2, which shows an experiment similar to Figure 1A. On this 60-fold faster time scale, the C trace appears almost linear except for an early portion where C increases more rapidly. It is clear from Figure 2B that the time derivative, averaged from 12 similar experiments, shows a transient component representing an early secretory burst. The secretory rate peaks at ~10 pF/s (9748 ± 741 fF/s, n = 16) and then falls 2- to 3-fold over the next 45 ms. An exponential fitted to the decline in the rate of secretion has a time constant of 14 ms. $[Ca^{2+}]_i$ measurements at 500 Hz (P.Thomas, J.G.Wong and W.Almers, to be published) show that at room temperature, Ca_i falls <10% between 15 and 100 ms after a flash under otherwise identical conditions, hence the decline of the rate of exocytosis in Figure 2B occurs while Ca_i remains essentially constant. We suggest that the decline represents the exhaustion of a finite pool of vesicles that can be released more rapidly than all others. After the secretory burst is over (40 ms), C has increased by 275 ± 20 fF (n = 16).

The increase in C begins within milliseconds after the

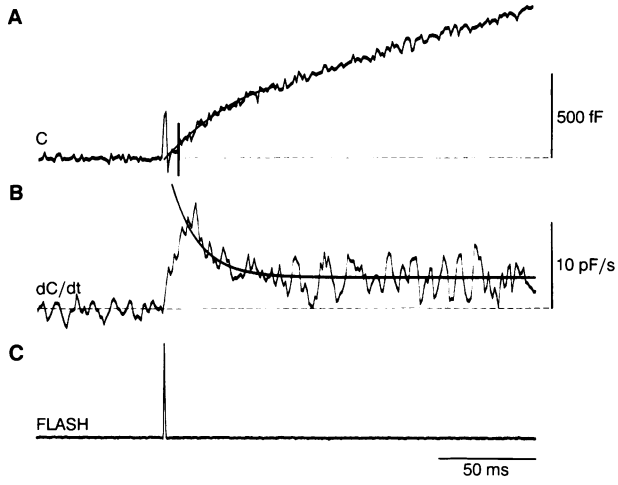

Fig. 2. Secretion begins within milliseconds. (**A**) C trace from an experiment similar to Figure 1A. A second-order polynomial was fitted to the 50 ms long segment of the trace beginning 7 ms (vertical line) after the flash; it intersected the abscissa 0.9 ms after the flash. The initial rate of secretion was taken as the slope of the fitted line at 7 ms. The amplitude of the early secretory burst was estimated from the mean increase in C within the first 30 to 50 ms after the flash. (**B**) Time derivative of the C trace, averaged from 12 cells. dC/dt was determined as for Figure 1 but with 7 ms long overlapping segments of the C trace. The continuous line is the sum of a declining exponential and a straight line. The exponential was fitted from 10–60 ms after the flash and the straight line from 60–190 ms after the flash. Note that the 7 ms interval used to calculate dC/dt artifactually lengthens the time taken for the trace to reach its peak. (**C**) Time-course of the flash measured with a photodiode.

flash. To make this more precise, a second-order polynomial was fitted to a 50 ms long segment starting 7 ms after the flash and extrapolated backwards. It fitted the trace well and intersected the abscissa at a time of 2.8 ± 0.6 ms after the flash (n = 16). Although significantly different from 0 (two-tailed Student's t-test, P <0.001), this time is at the limit of our resolution (one C measurement every 1.25 ms). We conclude that secretion begins within 3 ms after the flash.

Discussion

We have monitored secretion in single cells by measuring the membrane capacitance, an assay of the membrane added to the cell surface by exocytosis. To convert capacitance changes into numbers and percent of vesicles released, we refer to published electron micrographs. In Figure 5 of Chronwall *et al.* (1988), the average vesicle diameter is 117 nm (n = 200) and there are 7.8 ± 1.0 vesicles/μm^2 of section corresponding to 67 vesicles/μm^3 (Thomas *et al.*, 1990). In plate 1 of Douglas and Taraskevich (1978), vesicles had a diameter of 162 ± 3 nm (n = 200) and numbered 5.9 ± 0.3 /μm^2, representing 36/μm^3. We take the average of the two sets of values. A 140 nm diameter spherical vesicle has a surface area of 0.06 μm^2 and its exocytosis adds 0.6 fF of capacitance. With 52 vesicles/μm^3, a spherical melanotroph contains 60 000 vesicles because the plasma membrane capacitance of 5.8 ± 0.3 pF (n = 16) indicates a surface area of 580 μm^2 and a volume of 1150 μm^3.

Our results show that a step increase in Ca_i triggers three successive phases of secretion. The first is a rapid secretory burst that lasts ~40 ms and attains a peak rate of 10 pF/s, corresponding to 17 000 vesicles/s. Since C increases by 275 fF in the first 40 ms, the secretory burst represents the release of about 450 vesicles, <1% of the total. A slower, but still transient, phase of secretion follows the rapid burst. From Figure 2B we estimate that this phase begins with a rate of 4000 fF/s (~7000 vesicles/s) and increases C by ~1600 fF, corresponding to 2700 vesicles or 5% of the total. Finally, secretion continues at an essentially constant rate of 300 fF/s or 500 vesicles/s. Future work will explore how these components depend on Ca_i (P.Thomas, J.G.Wong and W.Almers, in preparation).

Since DM-nitrophen binds Mg^{2+} with high affinity and no Mg^{2+} was added through the patch pipette, the experiments in Figures 1A and 2 were carried out with an essentially Mg^{2+}-free cytosol. Most ATPases, in particular motor proteins such as kinesin and myosin, require Mg^{2+}–ATP as a substrate and hence are not expected to operate. Indeed, omitting ATP from the patch pipette did not strongly affect the capacitance changes following a step increase of Ca_i (data not shown). The vesicles whose exocytosis caused the increase in C observed here were probably not therefore actively transported during the times of our measurements. They must have been within diffusional distances of the plasma membrane before Ca_i increased.

It is tempting to speculate that at least the 450 vesicles comprising the early burst are already molecularly docked beneath the plasma membrane before Ca_i increases. This idea would also fit with the finding that they are exocytosed much more rapidly than all others. In this regard, they are functionally equivalent to the vesicles docked at the active zone of a synapse. Indeed, in chromaffin cells most secretory vesicles are excluded from the cell periphery by a 200–400 nm thick band of actin filaments, but ~450 vesicles (1.5% of the total) are found close to the plasma membrane (Burgoyne *et al.*, 1982; Burgoyne, 1991). If the vesicles comprising the early burst represent a distinct population primed for rapid exocytosis, then the time constant with which the secretory burst declines (14 ms) suggests an overall rate constant of 1000/14 = 71 s^{-1} for the exocytic reaction at 30–34°C. This is less than at nerve terminals (>1000 s^{-1} at 18–22°C; Katz and Miledi, 1965; Llinás *et al.*, 1981), but would still be rapid enough to allow hormone release to be temporally correlated with single action potentials which occur at a frequency of 1–2/s in melanotrophs (Douglas and Taraskevich, 1978). In chromaffin cells, another cell secreting dense-core vesicles, the average delay between Ca^{2+} entry and exocytosis of single vesicles is 51 ms, suggesting a rate constant of 20 s^{-1} at 22 –30°C (Chow *et al.*, 1992).

Morphology suggests that all of the 3000 vesicles exocytosed in the first two secretory phases may have been extremely close to the plasma membrane before the flash. In Figure 5 of Chronwall *et al.* (1988), 25 μm of plasma membrane could be clearly distinguished; 40 vesicles or 1.6/μm, had their centres within 140 nm of the plasma membrane. In plate 1 of Douglas and Taraskevich (1978), two cells had a combined circumference of 79 μm and had 56 vesicles or 0.7/μm within 140 nm of the cell surface. A typical 80 nm thin section will sample vesicles from an 140 + 80 = 220 nm thick layer if the vesicle diameter is 140 nm. Thus, a 140 nm thick shell just beneath the plasma membrane will contain (1/0.22 μm)×1.15/μm or 5 vesicles/μm^2. A typical melanotroph of 580 μm^2 surface area therefore has 2900 vesicles that are separated from the plasma membrane by one vesicle radius or less.

In conclusion, rapid measurement of exocytosis from single cells combined with rapid, uniform changes in Ca_i identify multiple components of secretion. Since our experiments were carried out in the absence of an effective substrate for molecular motors or other ATPases required for sustained secretion (Baker and Knight, 1978), the components identified here probably correspond to the Mg^{2+}-ATP-independent secretion observed previously (Reynolds *et al.*, 1982; Howell *et al.*, 1987; Holz *et al.*, 1989). Hence they represent late steps in the secretory pathway, such as fusion and possibly docking. In combination with pharmacological and molecular techniques, the approach described here allows study of the elements involved in regulating these late steps.

Materials and methods

Melanotrophs

Melanotrophs were obtained by enzymatic dissociation of rat pituitary intermediate lobes and maintained in primary culture for 2–10 days before use (Thomas *et al.*, 1990; Stack and Surprenant, 1991). Experiments were carried out at 30–34°C in an extracellular solution containing 125 mM NaCl, 20 mM TEACl, 10 mM sodium–HEPES, 5.5 mM glucose, 3 mM KCl, 2 mM $CaCl_2$ and 1 μM tetrodotoxin, pH 7.4. Single melanotrophs were voltage clamped in the whole cell mode (Hamill *et al.*, 1981). The intracellular solution applied through glass micropipettes contained 108 mM caesium glutamate, 16 mM TEACl, 2 mM Na_4ATP and 8 mM caesium–HEPES, pH 7.2. To measure and raise Ca_i, we also included 10 mM Na_4DM-nitrophen (Calbiochem), 8.5 mM $CaCl_2$, plus either 0.1 mM furaptra (Molecular Probes), a fluorescent Ca^{2+} indicator of low affinity (Raju *et al.*, 1989; Konishi *et al.*, 1991; Lattanzio and Bartschat, 1991), or 0.1 mM fura-2, an indicator of higher affinity (Grynckiewicz

et al., 1985). Glass micropipettes filled with these solutions had resistances of 1–2 MΩ and made 2–5 MΩ connections with the cytosol.

Measurement of Ca_i

To measure Ca_i, fluorescence was excited with pairs of 16 ms exposures to 340 and 380 nm light. $[Ca^{2+}]$ was calculated (Almers and Neher, 1985; Grynkiewicz *et al.*, 1985) from the fluorescence ratio, R (short wave/long wave) by:

$$[Ca^{2+}] = K^* \times (R - R_{min})/(R_{max} - R) \quad (1)$$

The calibration constants R_{min}, R_{max} and K^* were determined as follows. For measurements with furaptra, R_{min} was taken as the average of 20 readings taken before the flash. This is justified because the Ca_i established by our Ca^{2+}–DM-nitrophen mixture (163 ± 15 nM, four measurements with fura-2) is negligible compared to the K_d of Ca^{2+}–furaptra in melanotrophs (20–30 μM at 22–25°C; P.Thomas, J.G.Wong and W.Almers, unpublished). R_{max} was the ratio measured in cells loaded with 105 mM potassium glutamate, 45 mM potassium–HEPES, 15 mM $CaCl_2$, 10 mM Na_4DM-nitrophen and 0.1 mM furaptra. K^* was calculated from equation (1) using the ratio measured in cells loaded with 56 mM diglycolic acid, 26 mM $CaCl_2$, 10 mM Na_4DM-nitrophen, 40 mM potassium–MOPS, 12 mM potassium glutamate, pH 7.1. The free $[Ca^{2+}]$ of this solution was estimated as 94 μM at 32°C based on measurements with a Ca^{2+}-selective electrode. For measurements with fura-2, R_{min} was obtained from cells loaded with 45 mM EGTA, 45 mM KGlutamate, 45 mM KHEPES, 10 mM Na_4DM-nitrophen, 0.1 mM fura-2, pH 7.0. R_{max} was obtained as for furaptra. K^* was calculated from the ratio measured in cells loaded with 50 mM EGTA, 25 mM $CaCl_2$, 50 mM potassium glutamate, 50 mM potassium–HEPES and 0.1 mM fura-2, pH 7.0. For this solution we calculated a free $[Ca^{2+}]$ of 321 nM at 32°C (Blinks *et al.*, 1982) from the stability constants of Martell and Smith (1974). DM-nitrophen was photolyzed with a flash from a xenon arc lamp (Hi-Tech Scientific Ltd, UK) that was reflected off a dichroic mirror (Oriel Optical Co.) to eliminate light of wavelength >420 nm.

Determination of plasma membrane capacitance

Plasma membrane capacitance (C) was determined with a two-phase lock-in amplifier, which by phase-locked integration, sampled C every sinusoidal cycle (frequency = 800 Hz) and provided a frequency reponse of 400 Hz. We corrected for phase lags introduced by our instrumentation and by the pipette–cell junction by temporarily placing in series with the bath a 1 MΩ resistor and by adjusting the phase angle until the resulting deflection on the C trace disappeared (Joshi and Fernandez, 1988).

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References

Almers,W. and Neher,E. (1985) *FEBS Lett.*, **192**, 13–18.
Augustine,G.J. and Neher,E. (1992) *J. Physiol.*, **450**, 247–271.
Baker,P.F. and Knight,D.E. (1978) *Nature*, **276**, 620–622.
Blinks,J.R., Weir,W.G., Hess,P. and Prendergast,F.G. (1982) *Prog. Biophys. Mol. Biol.*, **40**, 1–114.
Breckenridge,L.J. and Almers,W. (1987) *Nature*, **328**, 814–817.
Burgoyne,R.D. (1991) *Biochim. Biophys. Acta*, **1071**, 174–202.
Burgoyne,R.D., Geissow,M.J. and Barron,J. (1982) *Proc. R. Soc. Lond., Ser. B*, **216**, 111–115.
Chow,R.H., von Rüden,L. and Neher,E. (1992) *Nature*, **356**, 60–63.
Chronwall,B.M., Bishop,J.F. and Gehlert,D.R. (1988) *Peptides*, **9 (Suppl. 1)**, 169–180.
Douglas,W.W. and Taraskevich,P.S. (1978) *J. Physiol.*, **285**, 171–184.
Fernandez,J.M., Neher,E. and Gomperts,B.D. (1984) *Nature*, **312**, 453–455.
Grynkiewicz,G., Poenie,M. and Tsien,R.Y. (1985) *J. Biol. Chem.*, **260**, 3440–3450.
Hamill,O.P., Marty,A., Neher,E., Sakmann,B. and Sigworth,F.J. (1981) *Pflügers Arch. Res. Physiol.*, **391**, 85–100.
Holz,R.W., Bittner,M.A., Peppers,S.C., Senter,R.A. and Eberhard,D.A. (1989) *J. Biol. Chem.*, **264**, 5412–5419.
Howell,T.W., Cockcroft,S. and Gomperts,B.D. (1987) *J. Cell Biol.*, **105**, 191–197.
Joshi,C. and Fernandez,J.M. (1988) *Biophys. J.*, **53**, 885–892.
Kaplan,J.H. and Ellis-Davies,C.R. (1988) *Proc. Natl. Acad. Sci., USA*, **85**, 6571–6575.
Katz,B. and Miledi,R. (1965) *Proc. R. Soc. Lond., Ser. B*, **161**, 483–495.
Kelly,R.B. (1991) *Curr. Opin. Cell Biol.*, **3**, 654–660.
Konishi,M., Hollingworth,S., Harkins,A.B. and Baylor,S. (1991) *J. Gen. Physiol.*, **97**, 271–301.
Lattanzio,J.F.A.,Bartschat,D.K. (1991) *Biochem. Biophys. Res. Commun.*, **177**, 184–191.
Lindau,M., Stuenkel,E.L. and Nordmann,J.J. (1992) *Biophys. J.*, **61**, 19–30.
Linstedt,A.D. and Kelly,R.B. (1991) *Curr. Opin. Neurobiol.*, **1**, 382–387.
Llinás,R., Steinberg,I.Z. and Walton,K. (1981) *Biophys. J.*, **33**, 323–351.
Martell,A.E. and Smith,R.M. (1974) *Critical Stability Constants. Vol.* 1, Plenum Press, NY, pp. 269–272.
Neher,E. and Marty,A. (1982) *Proc. Natl. Acad. Sci. USA*, **79**, 6712–6716.
Raju,B., Murphy,E., Levy,L.A., Hall,R.D. and London,R.E. (1989) *Am. J. Physiol.*, **256**, C540–C548.
Reynolds,E.E., Melega,W.P. and Howard,B.D. (1982) *Biochemistry*, **21**, 4795–4799.
Roberts,W.M., Jacobs,R.A. and Hudspeth,A.J. (1990) *J. Neurosci.*, **10**, 3664–3684
Stack.J. and Surprenant,A. (1991) *J. Physiol.*, **439**, 37–58.
Thomas,P., Surprenant,A. and Almers,W. (1990) *Neuron*, **5**, 723–733.

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